

## Appendix D



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## Recombinant Technology

# Functional human monoclonal antibodies of all isotypes constructed from phage display library-derived single-chain Fv antibody fragments

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## Abstract

We have constructed a series of eukaryotic expression vectors that permit the rapid conversion of single chain (sc) Fv antibody fragments, derived from semi-synthetic phage display libraries, into intact fully human monoclonal antibodies (mAb) of each isotype. As a model, a scFv fragment specific for sheep red blood cells (SRBC) was isolated from a semi-synthetic phage antibody (Ab) display library, and used to produce human mAbs of IgM, IgG1–IgG4, IgA1, IgA2m(1) and IgE isotype in vitro in stably transfected cells. N-terminal protein sequence analysis of purified immunoglobulin heavy (H) and light (L) chains revealed precise proteolytic removal of the leader peptide. Biochemical analysis of purified recombinant human mAbs demonstrated that properly glycosylated molecules of the correct molecular size were produced. The IgG and IgA mAbs retained SRBC-binding activity, interacted with different Fc receptor-transfectants, and induced complement-mediated hemolysis and Ab-dependent phagocytosis of SRBC by neutrophils in a pattern consistent with the immunoglobulin (Ig) H chain isotype. We conclude that in vitro produced recombinant human mAbs constructed from phage display library-derived scFv fragments mirror their natural counterparts and may represent a source of mAbs for use in human therapy. © 2000 Elsevier Science B.V. All rights reserved.

**Keywords:** Isotype switching; Phagocytosis; Complement-mediated lysis; Cloning vectors; Transient and stable expression

**Abbreviations:** Ab, antibody; BHK, baby hamster kidney cells; C, constant; complement; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; H, immunoglobulin heavy chain; Ig, immunoglobulin; L, immunoglobulin light chain; mAb, monoclonal antibody; NHS, normal human serum; scFv, single-chain Fv fragment; SRBC, sheep red blood cell; V, variable; V<sub>H</sub>, immunoglobulin heavy chain variable region; V<sub>L</sub>, immunoglobulin light chain variable region.

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## 1. Introduction

Since the advent of hybridoma technology, the potential of murine mAbs for therapeutic application in humans has been a focus of interest.

Clinical testing has shown that murine antibodies may evoke an anti-mouse immune response, the occurrence and magnitude of which is likely affected by the disease-related immune status of the recipient

and by the mode and duration of antibody administration (Jaffers et al., 1986). Although mouse antibodies of various isotypes, compared to their human equivalents, less efficiently bind human complement and Fc receptors expressed by human effector cells, some mouse antibodies have been shown to be cytotoxic in humans (Bonnefoy-Berard and Revillard, 1996). Yet, it may be anticipated that antibodies with human Fc regions are more effective cytotoxic reagents in human immunotherapy.

Attempts to generate mAbs of human origin using a variety of methods including somatic hybridization or immortalization of cells with Epstein–Barr virus have met with considerable technological and ethical problems and have not resulted in the establishment of reliable and robust methods. Humanization of murine mAbs by replacing Ig constant (C) or variable (V) region sequences, resulting in 'chimeric' and 'humanized' Abs (Morrison et al., 1984; Jones et al., 1986), has led to a reduction of the immunogenicity of the molecules. Further reduction of immunogenicity can be expected from completely human mAbs, which have recently been derived from transgenic mice and from phage display libraries.

Immunization of transgenic mice harboring human Ig H and L chain mini-loci and subsequent immortalization of spleen cells by somatic hybridization yields high-affinity human mAbs of predefined specificities (Taylor et al., 1992; Mendez et al., 1997). An alternative approach has been the construction of libraries of human Ab fragments expressed on the surface of filamentous phages and the selection of desired Ab-specificities by *in vitro* panning (Burton and Barbas, 1994; Winter et al., 1994). The Ab fragments obtained from these libraries are small scFv or Fab fragments and differ from intact natural Abs in terms of valency, pharmacokinetic behavior, and ability to recruit Fc region-dependent effector functions.

We have previously described the construction of a semi-synthetic phage Ab display library of scFv fragments and shown that a variety of phage selection procedures on purified antigens, tissue fragments, microorganisms, and eukaryotic cells permit the isolation of specific scFv Ab fragments (de Kruif et al., 1995a,b, 1996; Boel et al., 1998). Here we describe the construction of eukaryotic expression

vectors for the rapid conversion of phage display library-derived scFv Ab fragments to intact human IgG1, IgG2, IgG3, IgG4, IgA1, IgA2m(1), IgM, and IgE Abs. The procedure involves two cloning steps and constructs can be transiently or stably expressed in eukaryotic cells. The combined experiments indicate that the *in vitro* produced Abs are correctly assembled and glycosylated. Intact human mAbs constructed from a phage display library-derived scFv specific for SRBC were shown to perform effector functions that correlated with their C region isotype.

## 2. Materials and methods

### 2.1. Construction of expression vectors

pBR322 plasmids containing the human  $\kappa$  L chain (Hieter et al., 1982) or H chain IgG1–4 C regions (C $\gamma$ 1–4) (Kirsch et al., 1982) were used as a template to append *Bam*HI, *Not*I and *Sma*I restriction sites to the C regions by PCR amplification (Table 1A,B). The amplified products were cloned into the pNUT expression vector (Palmiter et al., 1987) using *Bam*HI and *Sma*I restriction enzymes, resulting in the plasmids pNUT-C $\kappa$  and pNUT-C $\gamma$ 1–4. Because the C $\gamma$ 1–3 C regions contain an internal *Sma*I restriction site, the amplified products were first digested with *Bam*HI and subsequently partially digested with *Sma*I, subjected to agarose gel electrophoresis and the full-length products were isolated and cloned into the pNUT vector.

The C regions of human IgM (C $\mu$ ) and IgA1 and IgA2m(1) (C $\alpha$ 1 and C $\alpha$ 2) (Ravetch et al., 1980) were PCR amplified from genomic clones in plasmid pBR322 using C $\mu$ - and C $\alpha$ -specific primers, respectively (Table 1A,B), digested with *Bam*HI and *Eco*RV and cloned into the *Bam*HI- and *Sma*I-digested pNUT vector.

A *Bam*HI restriction fragment containing the human IgE C region gene (C $\epsilon$ ) was obtained from a  $\lambda$  charon 38A phage clone (Max et al., 1982) and subcloned into the pBluescript vector (van der Stoep, 1995). C $\epsilon$  was PCR amplified from this plasmid with the C $\epsilon$  primers introducing *Not*I and *Eco*RV restriction sites (Table 1A,B), and cloned into the pNUT-

Table 1  
Oligonucleotide primers<sup>a</sup>

(A) Human back primers for immunoglobulin constant regions	
C <sub>κ</sub> -5	TTTATTAAAGGATCCGCGGCCGCTAGGAAGAACTCAAAAC
C <sub>κ</sub> -5	CAGGTGCGGATCCAGCGGCCGAGCCAGACACTGGAC
C <sub>μ</sub> -5	CAGCCCTGGGATCCAGGGCACCGCGCGCAGCTCCTCAC
C <sub>μ</sub> -5	CAATCATAAGGATCCCTCAGCGGGCCGCTCTGTGCTGGGTTCT
C <sub>μ</sub> -5	CAGGCTGCGGCCGCTGGCCTOAG
(B) Human forward primers for immunoglobulin constant regions	
C <sub>κ</sub> -3	TGGGGGCCCGGTACCTCTAACACTCTCCCT
C <sub>κ</sub> -3	GGGGCTTCCCGGTACCGCACTCATTTACCCGGAGA
C <sub>μ</sub> -3	CCGAGCCTGATATCAGGGTACCAGGGTCAGTAG
C <sub>μ</sub> -3	CAATATCGGATATCAGGTACCTCAGTAGCAGGTGCCGAC
C <sub>μ</sub> -3	AGGGAGGGGATATCAGGTACCTCATTTACC
(C) Primers to generate the HAVT20 leader	
HAVT20	CCCATAGAGGAATTCGGATCCAATGGCATGGCTTGCTTC
	CTGTGGGCACTTGTGATCTCCACCTGTCTGAATTTTCCA
	TGGCTGAAATTGAGCTCGTCGACAGGTGAGTCCGGCCG
	AAGCTTAAAGGTCTGG
	CCAGACCTTTAAGCTTGCGG
HAVT20-C	
(D) Human V <sub>κ</sub> back primers	
V <sub>κ</sub> 1a	TGTGACATCGAGCTCACCCAGTCTCCATCC
V <sub>κ</sub> 2a	TGTGATGTTGAGCTCACTCAGTCTCCATC
V <sub>κ</sub> 3pan	GA(A/T/C)AT(T/C)GAGCTCAC(G/A/T/C)CAGTCTCC
V <sub>κ</sub> 4	TGTGACATCGAGCTCACCCAGTCTCCAGACTCC
(E) Human J <sub>κ</sub> forward primer	
J <sub>κ</sub> pan	TTCTCGACTTGCGGCCGCAAGTGCACTTACGTTTGATCTCCACCTG
(F) Human V <sub>λ</sub> back primers	
V <sub>λ</sub> 1	TCCAGTCTGAGCTCACGCAGCGCCCTC
V <sub>λ</sub> 2	TCCCAGTTGAGCTCACTCAACCGCCCTCTG
V <sub>λ</sub> 3a	TCCTCTATGAGCTCACTCACCCACCT
V <sub>λ</sub> 3b	TCCTCTATGAGCTCACTCAGGACCT
(G) Human J <sub>λ</sub> forward primer	
J <sub>λ</sub> pan	TTCTCGACTTGCGGCCGCACTCACCTAGGACGGTCAGCTTGGTC

<sup>a</sup> Restriction sites used for cloning are underlined.

γ4 vector from which the γ4 chain was removed by restriction digestion with *NotI* and *SmaI*.

The pLEADER vector was constructed by extension of two partially complementary oligonucleotides, HAVT20 and HAVT20C (Table 1C), and cloning of the double-stranded product into the pUC18 vector using *EcoRI* and *HindIII*.

The seven V<sub>L</sub> genes used previously to construct the phage display library of scFv fragments (de Kruif et al., 1995a), were PCR amplified with the relevant primers (Table 1D–G) to introduce *SacI* and *NotI*

restriction sites and a splice donor site, and cloned into the pLEADER vector (Figs. 1A and 2). The V<sub>L</sub> gene fused to the HAVT20 leader was subsequently cloned into the pNUT-Cκ vector using *BamHI* and *NotI*.

The phagemid containing the coding sequence for the anti-SRBC scFv S6 was isolated from *Escherichia coli* XL1-blue cells (Stratagene, La Jolla, CA) and purified using a Qiagen plasmid isolation kit (Qiagen, Hilden, Germany). The phagemid was digested with *NcoI* and *XhoI* and the V<sub>H</sub> region

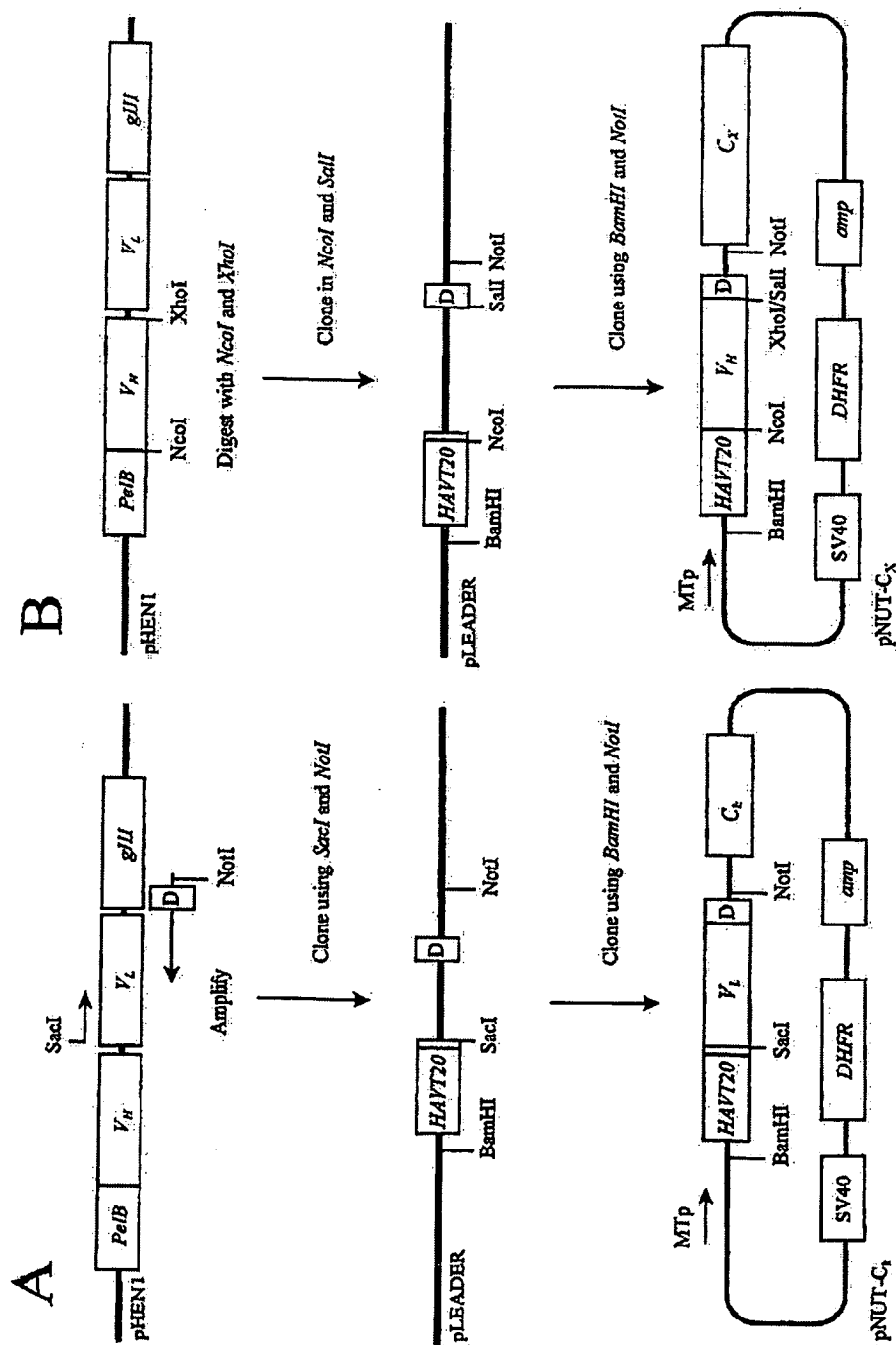


Fig. 1. Expression vectors for human Ig H and L chains and strategy for converting scFv fragments into intact human mAbs. (A) The  $V_L$  region is PCR-amplified to introduce restriction sites and a splice donor sequence (D) and cloned into the pLEADER vector. The  $V_L$  region, fused to the HAVT20 leader sequences is excised and subcloned into the pNUT- $C_k$  vector. (B) The  $V_H$  region is excised from the pHEN1 phagemid, cloned into pLEADER, and subcloned together with the HAVT20 leader and splice donor sequence into pNUT- $C_k$  containing the different H chain C regions. Amp, ampicillin resistance gene; DHFR, dihydrofolate synthetase gene; gIII, M13 minor coat protein III gene; Mbp, human metallothionein promoter; SV40, SV40 origin of replication.

EcoRI BamHI					HAVT20 leader							
<u>GAGGAATTCGGATCCA</u>					ATG	GCA	TGC	CCT	GGC	TTC	CTG	TGG
					met	ala	cys	pro	gly	phe	leu	trp
					HAVT20 leader							
GCA	CTT	GTG	ATC	TCC	ACC	TGT	CTT	GAA	TTT	TCC	ATG	
ala	leu	val	ile	ser	thr	cys	leu	glu	phe	ser	met	
		VI		SacI		Sall		NotI		HindIII		
<u>GCT</u>	<u>GAA</u>	<u>ATT</u>	<u>GAG</u>	<u>CTC</u>	<u>GTCGAC</u>		<u>AGGTGAGT</u>	<u>GCGGCCGCA</u>	<u>AAGCTT</u>			
ala	glu	ile	glu	leu								

20-PBS, and a 1/1000 dilution of horseradish peroxidase (HRP)-labeled Ab specific for the relevant human isotype was added. The plates were incubated for 1 h at 37°C and washed with Tween 20-PBS. HRP activity was quantified by addition of tetramethylbenzidine free base substrate and further incubation for 10 min at room temperature. The enzyme reaction was stopped with  $H_2SO_4$  and the  $OD_{450}$  was determined.

## 2.6. Purification of human Abs

The transfected cell lines were cultured in serum-free CHO-S-SFM II medium (Gibco-BRL, Grand Island, NY) and culture supernatants were harvested twice weekly. Cellular debris was removed from all supernatants by centrifugation at 3000 rpm for 10 min followed by filtration over a 0.2- $\mu$ m membrane (Gelman Sciences, Ann Arbor, MI).

All Ig subclasses were purified from culture medium by protein A-Sepharose chromatography (Pharmacia, Uppsala, Sweden). For every purification a new column was used to avoid cross-contamination of different Abs. The concentration of purified mAbs was determined using radial immunodiffusion plates

of the relevant isotype (LC-Partigen; Behringwerke, Marburg, Germany) according to the manufacturer's recommendations.

## 2.7. SDS-PAGE and N-terminal amino acid sequence analysis

Aliquots of the purified Abs containing 3  $\mu$ g of protein were run on a 10% reducing or 7.5% non-reducing SDS-polyacrylamide gel. Proteins were detected by Coomassie brilliant blue staining. For N-terminal amino acid sequence analysis, proteins were blotted onto a Problott membrane (Applied Biosystems, Foster City, CA) using 10 mM CAPS–10% methanol as blotting buffer. H and L chains were detected by Coomassie brilliant blue staining, and sequenced by Edman degradation.

## 2.8. Glycosylation analysis

Glycosylation of recombinant human Abs was determined following the same procedure as described for the isotype-specific ELISA, except that

FITC-labeled lectins were used in the detection step. After washing, individual wells were filled with PBS and bound FITC was quantified using a fluorescence multi-well reader (Cytofluor; Perspective Biosystems, Framingham, MA). The following lectins were used: *Limax flavus* (LFA) for the detection of sialic acid, *Erythrina cristagalli* (ECA) for galactose( $\beta$ 1,4)*N*-acetylglucosamine, and *Canavalia ensiformis* (Con A) for  $\alpha$ -D-mannose,  $\alpha$ -D-glucose, branched mannose (EY Laboratories, San Mateo, CA).

## 2.9. Binding of human Abs

SRBC were sensitized with the different purified S6 Ab preparations by incubation for 30 min at 37°C under agitation and washed in RPMI 1640 medium containing 5% FCS. SRBC-bound S6 mAbs were detected with FITC-labeled Abs specific for human IgG or IgA (Southern Biotechnology Associates) by incubation for an additional hour at 37°C, followed by flow cytometric analysis on a FACScan.

## 2.10. Fc receptor-transfected cell lines and SRBC-Ig rosetting assay

Murine B cell line IIA1.6 transfected with Fc $\gamma$ RI (van Vugt et al., 1996), Fc $\gamma$ RIIa-H131, Fc $\gamma$ RIIa-R131 (van den Herik-Oudijk et al., 1994), Fc $\gamma$ RIIb-NA2 (Ory et al., 1991) and Fc $\alpha$ RI (Morton et al., 1995) were cultured in RPMI 1640, supplemented with 10% FCS, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate. Rosette formation between Fc receptor-transfected cell lines and Ab-sensitized SRBC was performed as described in detail elsewhere (van de Winkel et al., 1987).

## 2.11. Fluorescent labeling of SRBC and phagocytosis

SRBC were stained with the red fluorescent dye PKH26 (Sigma) as described in detail elsewhere (Van Amersfoort and Van Strijp, 1994). Heparinized blood was obtained from healthy volunteers and neutrophils were isolated as described previously (Troelstra et al., 1997).

PKH26-labeled SRBC were sensitized with the

different S6-Ab preparations by incubation at 37°C for 30 min under mild agitation. Neutrophils and sensitized SRBC were washed and resuspended in RPMI 1640 containing 1% BSA to a final concentration of  $1 \times 10^7$  and  $1.2 \times 10^8$  cells/ml, respectively. Twenty- $\mu$ l aliquots of neutrophils and SRBC were mixed, centrifuged at  $25 \times g$  for 5 min at 4°C, incubated at 37°C for 20 min, washed and resuspended in 150  $\mu$ l PBS. Flow cytometric analysis was performed on a FACScan. Neutrophils were differentiated from free SRBC on the basis of their forward and side scatter properties.

### 2.12. Complement-mediated hemolysis

Complement-mediated hemolysis (CH50) was estimated by hemolytic microtiter assay as described previously (Klerx et al., 1983). To reduce background, NHS was preabsorbed with SRBC by mixing one volume of serum with three volumes of packed SRBC. After incubation for 20 min on ice, SRBC were removed by centrifugation and serum was stored in small aliquots at  $-70^\circ\text{C}$ .

## 3. Results

### 3.1. Construction of vectors for the production of human IgG1-4, IgA1-2, IgM, and IgE mAbs

We have constructed a series of vectors that allow the rapid conversion of scFv Ab fragments isolated from a semi-synthetic phage display library, to complete human mAbs of every isotype. The previously described phage Ab display library of scFv fragments was constructed from seven different  $V_L$  genes and more than  $10^8$   $V_H$  genes (de Kruif et al., 1995a). The  $V_H$  gene encoding a scFv isolated from this library was first cloned into the pLEADER vector, resulting in the introduction of a splice donor sequence at the 3' end and the eukaryotic HAVT20 leader peptide sequence at the 5' end of the  $V_H$  segment (Kimura et al., 1987; Kabat et al., 1991). The T cell receptor leader peptide HAVT20 was selected because it allowed the introduction of a *NcoI* restriction site at precisely the same position as was used in the pelB leader of the phage display library, without altering the amino acid sequence

(Hoogenboom et al., 1991). Part of the genomic human  $J_H4$  mini gene sequence (Kabat et al., 1991) was introduced downstream of a *SalI* restriction site in the pLEADER vector, to complete the  $V_H$  region and to introduce a splice donor sequence. The  $V_H$  region was subcloned into pLEADER using *NcoI* and *XhoI*, the same restriction sites which were used to construct the library (de Kruif et al., 1995a). Subsequently, using the *BamHI* and *NorI* restriction sites, the HAVT20- $V_H$ -splice donor construct was subcloned into the different expression vectors harboring the genomic H chain C regions, in which splice acceptor sequences are present (Fig. 1B).

The seven  $V_L$  genes present in the phage display library were PCR-amplified with primers introducing a splice donor site and restriction sites for cloning. To facilitate the cloning of  $V_L$  genes, the HAVT20 leader sequence is followed in frame by the sequence for the first four amino acids of a  $V_L$  domain containing a *SacI* restriction site, identical to that used in the construction of the phage display library (de Kruif et al., 1995a). The 3'-primer completes the  $V_L$  genes and adds part of the intron containing the splice donor sequence, and a restriction site. All  $V_L$  constructs were cloned into the pNUT-C $\kappa$  vector (Fig. 1A). Thus, all L chains will contain a human  $\kappa$  C region.

The expression vectors are derivatives of pNUT, which contains a dihydrofolate reductase gene for selection of stably transfected cells and a SV40 origin of replication for transient expression in COS cells (Fig. 1).

### 3.2. Selection of SRBC-binding phage

Phages were selected for binding to SRBC, and after three rounds of selection monoclonal phage preparations were tested by flow cytometry. Clone S6 was specific for SRBC and did not bind to ox or human erythrocytes or sheep leukocytes (not shown). The  $V_H3$  H chain and  $V_L3$  L chain genes encoding scFv fragment S6 were cloned into the different expression vectors.

### 3.3. Expression and purification of recombinant mAbs

Integrity of the constructs was assessed by tran-

sient expression in COS cells. Supernatants were harvested 3–5 days after transfection and analysed by ELISA for the presence of Abs, and by flow cytometry for binding to SRBC (not shown). After construct integrity was confirmed, stably transfected cell lines were established by co-transfection of H and L chain constructs in fur-BHK21 cells. Fur-BHK21 cells contain the furin gene and have been shown to be superior in the production of complex, high-molecular weight proteins such as factor VIII protein (Lankhof, 1996).

Methotrexate-resistant clones were analysed for Ab production and stable cell lines were propagated in selection medium. Culture supernatant was harvested twice weekly and all subclasses were purified from pooled supernatants using a protein A column, based on the observation that protein A binds to human V<sub>H</sub>3-encoded Ab domains (Sasano et al., 1993). MAb yields after purification as measured by radial gel filtration ranged from 5 to 18 mg/l of culture supernatant for the IgG subclasses, 0.5 to 3 mg/l for the IgA subclasses, 70 µg/l for IgM and 9.6 µg/l for IgE (Table 2). Because of their low yield, the IgM and IgE mAbs were not included in the functional studies.

#### 3.4. Characterization of recombinant human S6 mAbs

All S6 mAbs produced were tested in ELISA for the presence of a  $\kappa$  L chain and for the isotype of the H chain. IgG1–4 subclass identity was confirmed using subclass-specific mAbs (not shown).

SDS-PAGE under-reducing conditions showed H and L chain bands of the predicted size; L chains displayed a band of 30 kDa molecular mass while H chains revealed bands ranging from 50 to 60 kDa molecular mass, dependent on the isotype (Fig. 3A).

SDS-PAGE under nonreducing conditions revealed a single band of approximately 150–160 kDa, corresponding to the size of monomeric IgG mole-

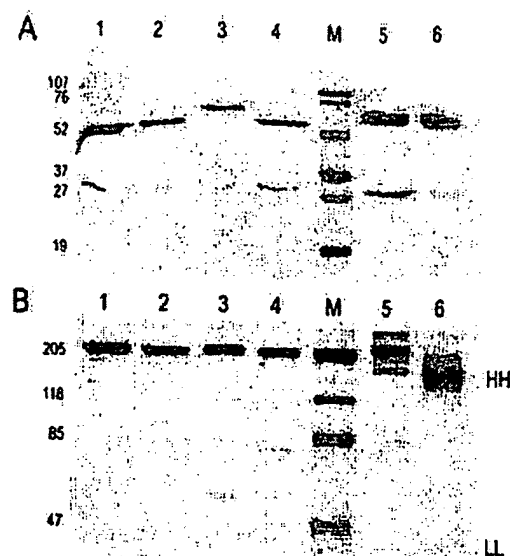


Fig. 3. Coomassie brilliant blue-stained SDS-PAGE gel of the purified S6 Ab preparations under reducing (A) and nonreducing conditions (B). HH and LL indicate homodimers of H and L chains, respectively, of IgA2m(1). Lane 1, IgG1; lane 2, IgG2; lane 3, IgG3; lane 4, IgG4; lane 5, IgA1; lane 6, IgA2m(1); M, molecular weight marker.

cules (Fig. 3B). In purified IgA1 preparations, a predominant band of 160 kDa was observed. In addition, a band of approximately 120 kDa molecular mass was visible, probably representing a complex of a single H and L chain, as has been reported previously (Morton et al., 1993). Some high-molecular weight aggregates were noticeable near the top of the gel. A faint band of 80–90 kDa, representing less than 10% of the total protein content, most probably represents a partial degradation product. Similar bands of 60 and 80 kDa were noticed in the IgG3 and IgG4 preparations, respectively.

Under nonreducing conditions, SDS-PAGE of purified IgA2m(1) preparations revealed two predominant bands of 120 and 50 kDa molecular mass,

Table 2  
Production levels of human immunoglobulins\*

IgG1	IgG2	IgG3	IgG4	IgA1	IgA2	IgM	IgE
17.9 mg	6.4 mg	13.3 mg	5.5 mg	3.0 mg	0.5 mg	70 µg	9.6 µg

\* Yield per liter after purification.



with some higher molecular weight aggregates. The 120 and 50 kDa bands represent disulfide-bonded H and L chain homodimers, respectively (Morton et al., 1993). In the IgA2m(1) allotype expressed here, H and L chain are not disulfide-linked. Instead, disulfide bonds are formed between the L chains (Mestecky and Kilian, 1985).

To verify the correct proteolytic removal of the HAVT20 leader peptide from the H and L chains, the N-terminal sequence of both chains from the S6-IgG4 Ab was determined. The amino acid sequences were precisely as predicted from the nucleotide sequences of the correctly cleaved  $V_H$  and  $V_L$  chains, EVQLVE and BIELT, respectively.

Human Ab molecules contain N-linked carbohydrates at conserved positions in the C regions of the H chains. Oligosaccharide is attached as high-mannose sugar and trimmed when the glycoprotein passes through the endoplasmatic reticulum and the *cis* Golgi. Processing may cease at this point, yielding glycoproteins with high-mannose sugars attached. Alternatively, processing may proceed to a complex biantennary form. The lectin Con A was used to confirm glycosylation, while the lectins ECA and LFA were used to detect the two terminal sugar residues of fully processed carbohydrates, galactose and sialic acid, respectively. S6-IgG1 and S6-IgG2 were analyzed using FITC-labeled lectins, and binding was quantified on a fluorometer. Protein A-purified IgG1 and IgG2 mAbs or crude culture supernatants from fur-BHK21 cell lines transfected with the S6-IgG1 or S6-IgG2 constructs were captured on microwells coated with anti- $\kappa$  L chain mAbs. Protein A-purified IgG1 and IgG2 mAbs displayed Con A reactivity, but lacked reactivity with ECA or LFA (not shown). In contrast, non-purified IgG1 and IgG2 present in culture supernatants bound Con A, ECA and LFA (not shown).

### 3.5. *In vitro* produced recombinant human mAbs are functional

The binding capacity of the different S6-Ab preparations was assessed by flow cytometry. The four human IgG and the two IgA isotypes revealed a similar pattern of binding to SRBC (Fig. 4). A control human IgG2 mAb, constructed from a phage library-derived scFv specific for group B streptococ-

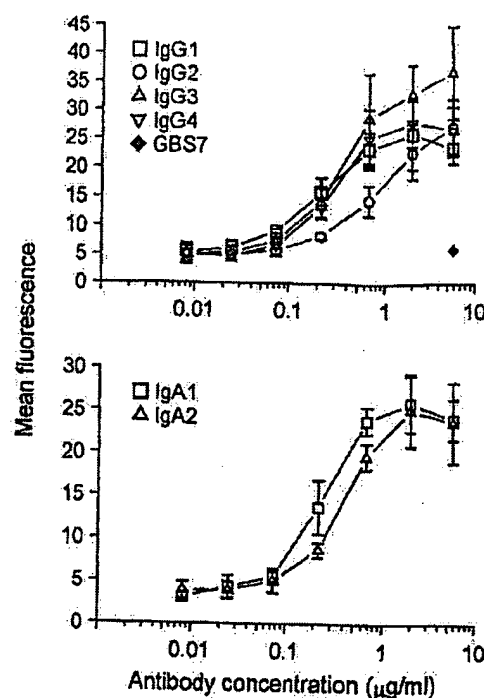


Fig. 4. Binding of IgG and IgA mAbs to SRBC. SRBC were sensitized with serially diluted preparations of the different mAbs and binding was detected with relevant FITC-labeled Abs by flow cytometry. Human mAb GBS7-IgG2, specific for group B streptococcal type III capsular polysaccharide, served as a control for background staining.

cal type III capsular polysaccharide (GBS7-IgG2, de Kruijff et al., 1995a) and produced and purified as described for the S6 mAbs, did not reveal any binding with SRBC (Fig. 4).

The interaction of recombinant S6 Ab with a panel of Fc receptor-transfected cell lines was determined using a SRBC-rosetting assay (Table 3). IgG1 and IgG3 were bound by all Fc $\gamma$  receptors, while IgG2-opsonized SRBC only formed rosettes with the Fc $\gamma$ RIIa-H131 transfected cell line. IgG4 was efficiently bound by Fc $\gamma$ RI, while some rosetting was observed with Fc $\gamma$ RIIa. IgA1 and IgA2 were the only isotypes bound by the Fc $\alpha$ RI-transfected cell line.

The capacity to mediate phagocytosis of SRBC by neutrophils was determined for the IgG1 and IgG4 anti-SRBC mAbs (Fig. 5). Neutrophils readily

Table 3  
Rosetting of Ig-opsonized SRBC with Fc receptor-transfected cells\*

	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2
FcγRI	100/100	0	100/100	100/100	0	0
FcγRIIa-H131	76/77	58/21	70/83	19/6	0	0
FcγRIIa-R131	80/85	0	100/60	47/6	0	0
FcγRIIb-NA2	62/65	0	41/27	0	0	0
FcεRI	0	0	0	0	85/78	21/7

\* Rosetting was defined as at least three SRBC bound to a cell. Two hundred cells per sample were scored and percentages are depicted for SRBC sensitized with 0.6/0.3 µg/ml antibody.

phagocytosed IgG1-opsonized SRBC, while SRBC incubated with IgG4 were not phagocytosed. For comparison fluorescence of neutrophils incubated with non-opsonized SRBC is shown (Fig. 5).

The ability to activate the classical pathway of

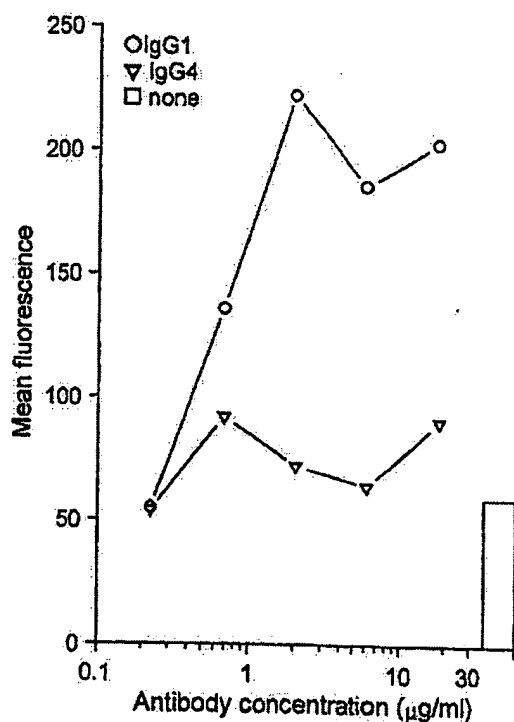


Fig. 5. Phagocytosis of IgG1- and IgG4-opsonized SRBC by neutrophils. PKH26-labeled SRBC were sensitized with different concentrations of the mAbs and after incubation with neutrophils, phagocytosis was measured by flow cytometry. The solid bar represents the level of background phagocytosis of non-opsonized SRBC.

complement, resulting in lysis of SRBC, was determined for a series of S6 mAb concentrations. The concentration of mAb that gave 50% lysis of SRBC (CH50) was calculated for the four IgG subclasses of S6-Ab (Fig. 6). Induction of hemolysis by IgG1 was very efficient, reaching optimal lysis at approximately 1 µg/ml of mAb. IgG3 also was capable of lysing SRBC, albeit slightly less efficiently than IgG1. IgG4 did not lyse SRBC at concentrations of up to 10 µg/ml, whereas marginal lysis was observed with

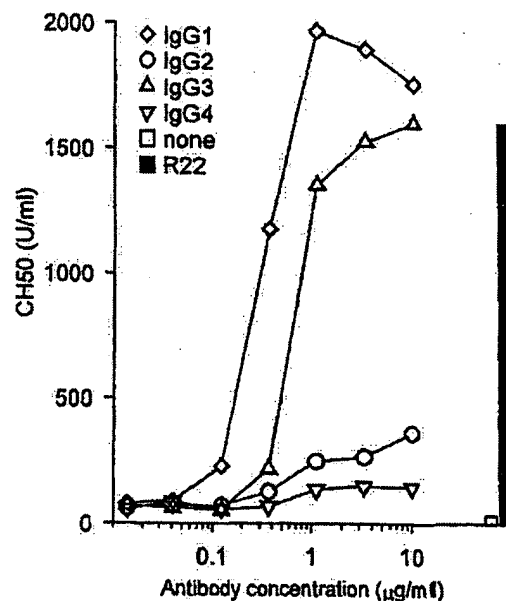


Fig. 6. Isotype-dependent, complement-mediated hemolysis. SRBC were sensitized with serially diluted mAb preparations and the amount of human serum required for 50% hemolysis was determined (CH50). As controls, non-opsonized SRBC and SRBC opsonized with serum from a hyperimmunized rabbit (R22) were included.

IgG2 at the highest mAb concentration tested (Fig. 6).

#### 4. Discussion

Phage Ab display technology has facilitated the rapid isolation of human Ab fragments specific for a wide variety of purified antigens, micro-organisms and eukaryotic cells. We have previously described the construction of a semi-synthetic phage Ab display library of scFv fragments and its applications (de Kruif et al., 1995a,b, 1996; Boel et al., 1998). Here we show that scFv selected from this library can be converted to complete and functional human mAbs of each of the eight isotypes. For the  $V_H$  region, the procedure was designed to encompass two cloning steps and avoids PCR amplification procedures and concomitant introduction of mutations. In the first step, the  $V_H$  region is cloned into the intermediate pLEADER vector to append the HAVT20 leader peptide sequence and a splice donor site. The T cell receptor  $\alpha$  chain leader HAVT20 (Kimura et al., 1987) was selected because it allowed the introduction of a *NcoI* restriction site facilitating the subcloning of the  $V_H$  chain, without interfering with the amino acid sequence. In addition, the pLEADER vector contains the genomic  $J_H4$  sequence downstream of a *SalI* restriction site to complete the  $V_H$  gene and to introduce the genomic splice donor sequence. In the second cloning step, the  $V_H$  region with appended leader and splice donor site is subcloned into one of eight expression vectors containing genomic sequences of each of the different human Ig C region genes. The genomic splice acceptor sequence resides in the C region domain constructs, resulting in the removal of the artificial intron between  $V_H$  and the C region by splicing.

The HAVT20 leader was also used for the construction of vectors for the expression of Ig L chains. A sequence encoding the first four amino acids of the  $V_L$  region, containing a *SacI* restriction site, was used in the phage library to facilitate cloning of  $V_L$  genes (de Kruif et al., 1995a). This same sequence was fused in frame to the HAVT20 leader sequence in the pLEADER vector. PCR amplification was used to complete the  $V_L$  domain and to introduce the genomic splice donor sequence and a *NotI* restriction

site for cloning (Fig. 1A). A set of seven L chain expression vectors containing each of the L chains used to assemble our phage Ab display library can be repeatedly used for expression of new human mAbs, based on scFv derived from this library.

Ig constructs were introduced in BHK21 cells transfected with the furin gene. Fur-BHK21 cells yield higher amounts of recombinant proteins as compared to BHK21 cells (Lankhof, 1996), probably because fur-BHK21 cells reach higher cell densities at confluency, resulting in increased protein accumulation in the supernatant. In addition, supernatants from fur-BHK21 cells may be harvested up to 8 weeks after reaching confluency. After purification, Ab yields of up to 18 mg/l of culture supernatant were obtained, comparing favorably to published production levels of recombinant human Abs that were not specifically selected for high levels of expression (Brüggemann et al., 1987; Co et al., 1992; Bender et al., 1993; Persic et al., 1997). Of note, IgM and IgE transfectants yielded considerably lower levels of Abs.

N-Terminal amino acid sequence analysis of the  $V_H3$  and  $V_{\lambda3}$  chains encoding Ab S6-IgG4, showed that the HAVT20 leader peptide was correctly removed from both the H and L chains in the mature Ab. The glycosylation pattern of recombinant mAbs produced in fur-BHK21 cells was analyzed using lectins specific for sugar residues attached in different stages of maturation of the oligosaccharide (reviewed in Wright and Morrison, 1997). MAb in the crude culture supernatant appeared to be completely glycosylated, resulting in oligosaccharides with terminal galactose and sialic acid residues. In contrast, the latter residues could not be detected on purified Abs obtained after protein A chromatography. It is likely that these residues were removed in the elution step with low pH buffers. Thus, fur-BHK-21 cells attach polysaccharides to the Ab molecules which are subsequently trimmed and processed to the complex biantennary form.

The IgG and IgA mAbs produced by fur-BHK21 cells assembled correctly as shown by SDS-PAGE analysis under nonreducing conditions (Fig. 3B). The IgA2m(1) allotype expressed here does not contain disulfide bonds between H and L chains; instead disulfide-bonded L chains dimers are formed (Mestecky and Kilian, 1985), which are linked to the

H chains by noncovalent interactions, explaining the predominant bands of 120 and 50 kDa molecular mass.

The different isotypes of the human Ab S6 bound to Fc receptors in a pattern consistent with published reports (van de Winkel and Anderson, 1991; Shen, 1992). IgG1 and IgG3 interacted with all Fc $\gamma$  receptors tested, whereas IgG2 was efficiently bound by the Fc $\gamma$ RIIa-H131 transfectant only. IgG4-sensitized SRBC bound to Fc $\gamma$ RI-transfected cells, but some rosetting with Fc $\gamma$ RIIa was also seen. The IgA1 and IgA2 preparations bound to Fc $\alpha$ RI-transfected cells only, and no IgG preparation bound to these cells. Phagocytosis of IgG1- but not of IgG4-opsonized SRBC is consistent with the absence or very low level expression of Fc $\gamma$ RI on resting neutrophils (Antal-Szalmas et al., 1997).

In our model, IgG1 induced complement-mediated lysis much more efficiently than IgG3. The relative efficiency of lysis of target cells by different Ig subclasses has been shown to be dependent on the target epitope. IgG1 is considerably more effective than IgG3 in mediating lysis of NIP-coated erythrocytes (Brüggemann et al., 1987; Bindon et al., 1988), while IgG3 is more effective at mediating lysis of dansyl-coated erythrocytes (Dangl et al., 1988). However, in another report using NIP-coated erythrocytes, IgG1 was better than IgG3 at high antigen concentration, while the reverse was observed at lower antigen concentration (Garred et al., 1989; Lucisano Valim and Lachmann, 1991). IgG2 was only effective at the highest antigen densities. This observation fits well with the known preferential IgG2 response against polysaccharides on encapsulated bacteria. The repetitive epitopes of polysaccharides would fulfil the requirements for complement activation found in these studies. Indeed, complement activation by the human IgG2 isotype has been reported for purified polyclonal Ab preparations directed against bacteria (Weinberg et al., 1986; Givner et al., 1987; Amir et al., 1990; Bredius et al., 1992). The recombinant S6 Abs recognize a naturally occurring antigen on SRBC, which, in Scatchard analysis, was estimated to be expressed at less than 3000 molecules per cell. Indeed, IgG2 induced some hemolysis only at the highest Ab concentrations tested, while maximum complement-mediated hemolysis was reached at low IgG1 and IgG3

concentrations. As expected, no hemolysis was observed with IgG4 at any of the Ab concentrations tested.

The conversion of phage Abs fragments to complete Ig molecules of a single isotype has been reported for a tetanus toxoid-specific human Fab fragment (Bender et al., 1993), two complement component C5a-specific murine Fab fragments (Ames et al., 1995), a HPA-1a-specific human scFv Ab (Watkins et al., 1999), and a human scFv Ab fragment of which the target was not specified (Persic et al., 1997). The Fab fragments and the HPA-1a-specific scFv fragments performed similarly to the corresponding whole Abs (Bender et al., 1993; Ames et al., 1995; Watkins et al., 1999), whereas the specificity and biological activity of the other scFv fragment and corresponding whole Ab have not been compared (Persic et al., 1997). The SRBC-specific scFv fragment described here was converted to intact fully human anti-SRBC Abs of all eight isotypes. The Abs retained binding specificity, were correctly assembled and glycosylated and performed in Fc receptor binding, complement activation and phagocytosis assays conform their Ig isotype. Human scFv fragments against a wide variety of antigens have been isolated from a number of combinatorial and semi-synthetic phage display libraries. Recently developed mutagenesis and selection strategies facilitate the construction of very high affinity derivatives with affinities in the picomolar range (Schier et al., 1996). The expression vectors described here facilitate the rapid construction of high affinity, intact fully human mAbs for clinical application using scFv Ab fragments selected from phage display libraries as building blocks.

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